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THE ROLE OF EYESTALK HORMONES IN VITELLOGENESIS DURING THE BREEDING SEASON IN THE CRAB, *PARATELPHUSA HYDRODROMOUS* (HERBST)

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Abstract

In *P. hydrodromous,* vitellogenesis is divided into two phases, Vitellogenesis I (V_1, subdivided into Stages 1–3) and Vitellogenesis II (V_2). The accelerated ovarian growth induced by bilateral eyestalk ablation during the first half of the breeding season apparently proceeded normally until Stage 3 of V_1. Subsequently, the yolk showed signs of impoverishment, particularly in its total protein and lipid contents. In both gross morphology and biochemistry, the ovaries of eyestalkless crabs in V_2 during the breeding season were closer to V_2 ovaries of normal crabs than to V_2 ovaries induced to develop in destalked crabs during the prebreeding season. The ovarian abnormality seen when eyestalk ablation occurs during the breeding season is not due to the unpreparedness of the oocyte population to begin vitellogenesis. In *P. hydrodromous,* the ovary, at all stages of its development except perhaps at late V_2, seems to depend on eyestalks for the normal maintenance of vitellogenesis. *P. hydrodromous* females do not spawn if eggs artificially induced to form are not normal. Influence of the eyestalk on spawning is not directly via the nervous system; an eyestalk hormone controlling oviposition, if present, is released at least one week prior to spawning.

Introduction

In many decapod crustaceans, bilateral eyestalk ablation ($-E_2$) leads to accelerated ovarian growth (Adiyodi and Adiyodi, 1970), but reports of successful spawning following such accelerated ovarian growth are few (Cheung, 1969; Hinsch, 1972). Our earlier investigations on *Paratelphusa hydrodromous* showed that the precocious ovarian growth induced by $-E_2$ during the prebreeding season (September–November), was abnormal; yolk accumulated in oocytes was biochemically impoverished and no eggs were spawned (Anilkumar and Adiyodi, 1980). Counts of avitellogenic and early vitellogenic oocytes in ovaries of *P. hydrodromous* during the prebreeding season suggested that the abnormality in ovarian physiology found in $-E_2$ females during the prebreeding season was at least in part due to the unpreparedness of a significant proportion of the oocytes to commence active vitellogenesis. This prompted us to study the effects of $-E_2$ on oviposition and the composition of yolk in crabs during the breeding season, when the oocytes are generally in a state of readiness to begin active vitellogenesis.

In the Calicut population of *P. hydrodromous,* breeding season and start of vitellogenesis begins in December. The period of vitellogenesis in *P. hydrodromous* has two phases, Vitellogenesis I (V_1) and Vitellogenesis II (V_2); the former is further subdivided into three stages (Adiyodi, 1968). During Stage 1 (V_1S_1) the ovary is whitish

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(oocyte diameter: 0.5–0.6 mm); oocytes assume a pale cream color by Stage 2 (V_1S_2) (diameter: 0.61–0.8 mm). As the ovary reaches Stage 3 (V_1S_3) the oocytes appear orange and 0.81–1.30 mm in diameter. V_2 ovaries are a deep orange with a tint of brown (oocyte diameter: 1.31–2.0 mm). The Calicut population of *P. hydrodromous* generally has a single spawning season, sometime during March/April. Eggs are carried by the female in the concavity of her broad abdomen; young are released at the monsoon onset (June). Premolt changes begin following release of the brood; molting occurs during June/July. August through November is the prebreeding season; individuals remain in intermolt and ovaries are white bands showing no signs of vitellogenic activity.

Materials and Methods

P. hydrodromous females used in this study were collected from rice farms around the University campus and cultured in laboratory tanks as previously described (Anilkumar and Adiyodi, 1980). Crabs were reared in cement cisterns laid with wet sand at the bottom and fed ox liver *ad lib.* Acclimation to the laboratory occurred for 3–4 days prior to the experiment.

Eyestalks were removed from intermolt adult females [3.8–4.2 cm carapace width (cw)] in December. Molt stages were determined by observing the pleopods: pleopods of intermolt crabs had only a single cuticular layer; the onset of premolt was marked by the appearance of a new cuticular layer in the pleopod (after Anilkumar, 1980). For each quantitative analysis, ovaries were dissected out (by cutting open the carapace) from groups of 5 destalked animals 15, 30, and 45 days after $-E_2$. Each time, ovaries from five control crabs were also dissected out and analyzed for comparison. The Student's *t*-test analyzed the level of significance of difference between the experimentals and controls. Experimental data on crabs in prebreeding season, used for comparison in this study, have been taken from Anilkumar and Adiyodi (1980).

One hundred and forty-seven adult female crabs were sacrificed to analyze the effect of destalking on ovarian physiology and spawning during the breeding season. Further, not less than 75 freshly caught animals were used each month (throughout one full annual cycle; sample size >900) for studies on seasonal changes in the ovary.

The ovary was homogenized with 5% trichloracetic acid (TCA) to precipitate protein which, after centrifugation, was dissolved in 0.1 *N* NaOH and quantitatively estimated after Lowry *et al.* (1951). To estimate lipid, dried tissue (ovary) was subjected to Soxhlet extraction using the solvent chloroform (Vogel, 1959). Carbohydrates of the tissue were separated into ethanol (80%)-soluble oligosaccharide fraction and ethanol-insoluble polysaccharide fraction (Johnston and Davies, 1972) and estimated by phenol-sulphuric acid method (Dubois *et al.*, 1956). Free amino acids (FAA) were extracted using 80% ethanol and estimated after Lee and Takahashi (1966). The pattern of distribution of oocytes in the ovary of both experimental and control groups was studied by making numerical counts of the yolky and yolkless oocytes; the ratio of yolky to yolkless oocytes in experimentals and controls was subjected to χ^2 test for comparison.

To evaluate the role of eyestalk hormone(s) on spawning, a series of experiments were conducted during the final phase of vitellogenesis (late V_2—March). Both eyestalks were removed from 13 adult females, out of which 6 (Group I) received regular injections of extracts of 2 eyestalks (in 0.9% saline) at a time, twice a week. A group of normal females (14) with intact eyestalks constituted the controls (Group II). Both experimentals and controls were reared in the laboratory for 11 days [during which time most of the individuals (19 out of 27) (70%)] did oviposit.

RESULTS

In December, $-E_2$ induced in crabs an initial phase of hyperphagia lasting 7–10 days; this was followed by a period of apparent normalcy in feeding pattern until 25–30 days postoperation, after which set in a phase of hypophagia lasting the duration of the experiment (45 days).

Ovarian response to $-E_2$ during the breeding season

At the beginning of the experiment in December (the first half of the breeding season of *P. hydrodromous* in Calicut), ovaries of experimentals and control crabs were in V_1S_1. $-E_2$ induced an acceleration in ovarian growth in both oocyte size (Table I) and ovarian wet weight (Table II). All experimental crabs (and controls) remained uniformly in intermolt even 45 days postoperation; a second cuticular layer was not formed in any of the experimentals showing a lack of initiation of premolt events (somatic growth).

The ovaries of experimental females which had reached V_1S_3 or V_2, 15 days post-operation (Table I), had a gross morphology comparable to that of normal females in comparable stages of ovarian development. By 45 days postoperation, the ovaries became abnormally brown and the oocytes assumed a rather flabby appearance as though they were only partially filled with yolk. The crabs become hypophagic at this time: the change in appearance of the ovary could be related, in part, to the paucity of alimentary organic resources. Chi-squared analysis, however, showed that the ratio of yolky to yolkless oocytes in the ovary of $-E_2$ females in V_2 was comparable to that of normal ovary in V_2 ($\chi^2 = 1.16$; $P > 0.2$).

A normal (control) crab required 30–45 days to reach V_1S_3 from the date of commencement of the experiment. Ovaries of destalked crabs, however, reached V_1S_3 by 15 days postoperation; in some instances the oocytes in $-E_2$ crabs were too precocious and had already reached V_2 by 15 days (Table I). Further, in experimentals, $-E_2$ led to a statistically significant precocious increase in total TCA-precipitable proteins, chloroform-extractable lipids, oligosaccharide fraction, polysaccharide fraction, and FAA content (Table II) of the ovaries, compared to controls. To evaluate whether the precociously incorporated yolk (after $-E_2$) is normal, we compared our present data with those of normal females with comparable ovarian stages (the latter from Anilkumar and Adiyodi, 1980). This (judged from the levels of organic constituents) revealed that the ovaries of $-E_2$ crabs responding precociously to the operation were normal until 15 days postoperation. The profiles of major vitelline components of $-E_2$ crabs at

TABLE I

Oocyte size and stage of the ovary after $-E_2$ during the breeding season in P. hydrodromous

Period after operation	Oocyte size in mm (O)		Stage of the ovary	
	Control (C)	Ablated ($-E_2$)	Control	Ablated
15 days (X)	0.69 ± 0.04	1.24 ± 0.44	V_1S_1 or V_1S_2	V_1S_3 or V_2
30 days (Y)	0.75 ± 0.05	1.55 ± 0.03	V_1S_1, V_1S_2, or V_1S_3	V_2
45 days (Z)	0.99 ± 0.05	1.91 ± 0.17	V_1S_3	V_2

X.O.C. < X.O. $- E_2$; $t = 2.41$ ($P < 0.05$)

Y.O.C. < Y.O. $- E_2$; $t = 13.77$ ($P < 0.01$)
Z.O.C. < Z.O. $- E_2$; $t = 5.2$ ($P < 0.01$)

TABLE II

Changes in ovarian wet weight and biochemical components 15, 30, and 45 days after $-E_2$ during the breeding season in P. hydrodromous *(weights shown in mg/100 g animal weight; mean ± S.E.)*

		15 days	30 days	45 days
Wet weight	Control	376.91 ± 19.80	726.34 ± 28.11	1172.08 ± 32.73
	Ablated ($-E_2$)	1630.07 ± 252.71	2998.21 ± 395.86	3513.35 ± 207.77
	$P<$	0.01	0.01	0.01
Total proteins	Control	42.35 ± 6.86	50.92 ± 7.03	257.18 ± 16.49
	Ablated ($-E_2$)	213.02 ± 31.40	370.29 ± 45.40	454.91 ± 60.09
	$P<$	0.01	0.01	0.02
Total lipids	Control	33.96 ± 5.38	52.79 ± 7.16	195.82 ± 30.42
	Ablated ($-E_2$)	225.16 ± 50.60	386.54 ± 25.30	508.38 ± 62.03
	$P<$	0.01	0.01	0.01
Oligosaccharide fraction	Control	6.08 ± 0.86	10.02 ± 2.57	11.21 ± 0.83
	Ablated ($-E_2$)	19.92 ± 3.19	22.08 ± 2.92	25.40 ± 3.87
	$P<$	0.01	0.01	0.01
Polysaccharide fraction	Control	3.68 ± 0.42	4.18 ± 0.79	4.69 ± 0.88
	Ablated ($-E_2$)	7.62 ± 1.50	10.58 ± 0.71	12.53 ± 2.08
	$P<$	0.05	0.01	0.01
Free amino acids	Control	5.16 ± 1.19	8.52 ± 1.31	10.07 ± 1.03
	Ablated ($-E_2$)	11.48 ± 1.67	21.33 ± 3.10	23.74 ± 4.88
	$P<$	0.02	0.01	0.05

P represents the level of significance of difference between the control and $-E_2$ ovaries.

V_1S_3 (15 days postoperation) were comparable (statistically) to those of a normal crab having V_1S_3 ovary. As the $-E_2$ ovaries passed from V_1S_3 to V_2 (by 30 days postoperation), however, this sense of normalcy was lost. Chief organic compounds of the yolk, particularly proteins and lipids, were impoverished in V_2 ovaries compared to normal V_2 ovaries ($P < 0.05$).

Effect of $-E_2$ on spawning

Final oocyte diameters in $-E_2$ crab ovaries often surpassed those of normal mature eggs, but none of the females, destalked in December, spawned. In contrast, normal controls maintained under identical culture conditions spawned during the normal spawning season.

In the second series of our experiments, in which adult females were destalked during the final phase of vitellogenesis (March), 66% (4 out of 6) of the Group I females, 79% (11 out of 14) of Group II females, and 57% (4 out of 7) of experimentals oviposited normally within 1 week postoperation.

DISCUSSION

If eyestalks are removed in December, oocyte growth is precipitated (Table I), the ovary gains weight, and there is a statistically significant rise in levels of different organic constituents of the ovary (Table II), all precociously in comparison to controls. This suggests that a gonad-restraining principle or principles (GIH) may be present in *P. hydrodromous* eyestalks during the breeding season. Earlier studies had shown that such a principle may also be present in eyestalks during the prebreeding season and that ovarian inactivity seen during this period is not due to ovarian refractoriness, but due to the presence of GIH in effective titres (Anilkumar and Adiyodi, 1980).

It is likely that the effects of $-E_2$, as we observed on *P. hydrodromous* ovaries,

could be related to increased production of ecdysteroids (ECD). Removal of eyestalks, which are also the sources of the molt-inhibiting hormone (MIH), could lead to activation of the Y-organ and to increased secretion of ECD. A vitellogenic function for ECD has been established in Diptera among insects (Hoffmann *et al.*, 1980 for review) and is indicated in amphipods, isopods, and natantian decapods among crustaceans (Kurup and Adiyodi, 1984). Further, multiple limb autotomy (MA) in crabs accelerates the onset of molting (Skinner and Graham, 1972; Kurup and Adiyodi, 1984) or vitellogenesis (Kurup and Adiyodi, 1984), depending upon the physiological phase of the animal at the time of MA. MA causes a dramatic increase in ECD levels in the hemolymph (McCarthy and Skinner, 1977), suggesting the possible involvement of ECD component in vitellogenesis. What is puzzling, however, is that $-E_2$ did not precociously initiate premolt events in any of our crabs even 45 days after $-E_2$. The problem of involvement of the ECD component in accelerating vitellogenesis can be finally settled only when data on ECD titers in hemolymph and the degree of responsiveness of the target tissues become available for this species.

Present experiments show that eyestalks are necessary to maintain normal ovarian growth during the breeding season. Ovarian physiology is apparently normal only for about 15 days following $-E_2$. During subsequent weeks, the ovary seems to lose its ability to support a normal and properly balanced vitellogenic process in the absence of eyestalks. This is clearly borne out by the difference in wet weight during the breeding season between V_2 ovaries of $-E_2$ females and V_2 ovaries of normal females and by the decrease in quantity of yolk (reflected in wet weight of the ovary) and in quantities of the chief organic constituents of yolk. In V_2 ovaries of $-E_2$ females (not controls), certain flabby oocytes often larger than normal mature eggs, but incompletely filled with yolk, are present. This indicates that accumulation of yolk within oocytes following $-E_2$ could be abnormal at least in one population of oocytes.

Comparison between V_2 ovaries of normal crabs and $-E_2$ crabs revealed that major organic components of yolk whose synthesis or accumulation in oocytes is most affected by $-E_2$ are proteins and lipids. The fall in organic reserves of the ovary, noticed in crabs 30 days postoperation, is unlikely to be related to lack of feeding, for $-E_2$ crabs do not cease feeding until 25–30 days postoperation. We do not rule out the possibility that metabolic derangements resulting from lack of food may, however, be responsible, at least in part, to the impoverished nature of the $-E_2$ crab ovary, 45 days postoperation. $-E_2$ may gradually render the oocyte synthetic machinery inefficient or hinder uptake of vitellogenin and utilization of lipid from blood by the ovary.

Abnormal ovarian physiology, seen during the breeding season in $-E_2$ crabs, cannot be explained as the consequence of a hasty initiation of the vitellogenic process in an otherwise unprepared ovary, for the operation was conducted at a time when the ovary was ready to begin normal vitellogenesis. The eyestalk is, therefore, *sensu stricto* not inhibitory, but possibly is a seat of some 'restraining factor', which seems to be essential to the normal progression of vitellogenesis. The question arises whether the precocious accumulation of yolk reserves and subsequent malfunction of the ovary consequent upon $-E_2$ is due to the absence in circulation of GIH and/or to some metabolic principles contained in eyestalks. As ovary is the target tissue involved, GIH appears to be the most likely candidate which restrains oocyte growth, but whose presence is, nevertheless, necessary for proper ovary function. It may be argued that the term GIH denotes not a single hormone, but a group of factors some of which may be necessary for normal differentiation of the oocytes and others to regulate the many metabolic processes that help maintain normal vitellogenesis. Eyestalks in decapod crustaceans contain factors influencing the metabolism of carbohydrates (Chang and O'Connor, 1983), lipids (Chang and O'Connor, 1983), and nitrogenous substances (Claybrook, 1983), but to what extent they specifically influence ovarian metabolism is unknown.

A comparison of the biochemical composition of V_2 ovaries of $-E_2$ females whose eyestalks were ablated during the prebreeding season (the data from Anilkumar and Adiyodi, 1980) with V_2 ovaries of $-E_2$ females destalked during the breeding season reveals that, despite the differences discussed above, the latter are closer to ovaries from intact female in V_2. Further, our previous analyses (Anilkumar and Adiyodi, 1980) revealed that during the prebreeding season ovarian abnormality resulting from $-E_2$ manifests itself from 15 days postoperation, when the ovaries are in V_1S_3, whereas during the breeding season (present study) such abnormality becomes evident only over a longer period. We do not know whether the difference in response of crabs destalked during the prebreeding season is directly or indirectly related to hemolymph protein levels (Anilkumar, 1980). One basic difference between crabs destalked during prebreeding or breeding seasons is the ratio of yolky to yolkless oocytes in ovaries. Experimental crabs destalked at the beginning of the breeding season have oocytes in V_1S_1, whereas ovaries of intact crabs at the beginning of the prebreeding season have not reached V_1S_1, and most of the oocytes have diameter less than 0.5–0.6 mm (Pillai and Adiyodi, unpub.). The uneven acceleration of vitellogenesis, apparent in ovaries of $-E_2$ females during the prebreeding season (Anilkumar and Adiyodi, 1980), is thus primarily a function of the age of oocytes at the time of eyestalk removal. Pillai and Adiyodi (unpub.) further observed that the oocytes of *P. hydrodromous* occupy their definitive positions in the ovary following the postspawning revival of activity of the germarium, but have to grow considerably (perhaps along with some cytological differentiation), before acquiring the characteristics of oocytes in V_1S_1. This was further substantiated in our laboratory by another series of experiments (Kurup and Adiyodi, in prep.). In *P. hydrodromous,* destalked during the postspawning period, the ovary responded by accelerated growth, but the oocytes did not grow beyond V_1S_2 or V_1S_3. Again, the pattern of oocyte response was uneven, only a few oocytes reached V_1S_2 or V_1S_3 (Kurup and Adiyodi, in prep.).

The ovary of *P. hydrodromous* seems to require eyestalk factors (possibly GIH) at all stages of its development except perhaps at the final phase of vitellogenesis (late V_2). When destalking was conducted during the postspawning season, only a few oocytes responded to $-E_2$ and even they did not grow beyond V_1S_2 or V_1S_3 (Kurup and Adiyodi, in prep.); few oocytes reached V_2 under $-E_2$ during the prebreeding season (Anilkumar and Adiyodi, 1980). Our present study reveals that during the breeding season the oocytes of $-E_2$ females reached V_2 much sooner, *i.e.,* by 30 days postoperation; at this time, however, ovarian biochemical impoverishment occurred, and the operated individuals could not successfully complete vitellogenesis. When eyestalk ablation was conducted during the final phase of vitellogenesis (March), however, most of the individuals could successfully complete vitellogenesis and spawning; the dependence on eyestalk (for maintenance of normalcy of ovarian development) apparently stops once oocytes reach late V_2.

Mating occurs in *P. hydrodromous* during late June–early August, between a soft postmolt female and a hard intermolt male. During the first breeding season following pubertal molt, young female *P. hydrodromous* do not commence vitellogenesis if mating does not take place, probably because there is no chance of fertilization (Krishnakumar and Adiyodi, in prep.). Successful mating is no guarantee that spawning will take place; spawning will not occur if ovarian normalcy is disturbed, for instance, by $-E_2$. Eyestalk influence, if any, on spawning is not via the nervous system; ablation of eyestalks during the final phase of vitellogenesis did not inhibit oviposition. MA, carried out during the reproductive phase, accelerated ovarian growth in *P. hydrodromous* but such females failed to oviposit (Kurup and Adiyodi, 1984). The same operation when conducted during the final phase of vitellogenesis (*i.e.,* 12–20 days

prior to oviposition) did not induce any perceptible acceleration in ovarian growth or interfere with the spawning process (Kurup and Adiyodi, 1984). The results reveal that a block to the release of eyestalk principle(s) either by $-E_2$ or MA in turn blocks spawning except perhaps in the final phase of vitellogenesis. We do not know whether the eyestalk is the source of a separate oviposition-inducing hormone as suggested by Bomirski and Klek (1974) in *Rithropanopeus harrisii*. Regardless, during our destalking experiments conducted in March, two of the eyestalkless animals oviposited only on the 7th day postoperation. We therefore suggest that if the eyestalks of *P. hydrodromous* are seats of oviposition-inducing hormone (as suggested in *R. harrisii*), the hormone is released at least one week prior to spawning.

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LITERATURE CITED

ADIYODI, R. G. 1968. Protein metabolism in relation to reproduction and moulting in the crab, *Paratelphusa hydrodromous* (Herbst): part I—Electrophoretic studies on the mode of utilization of soluble proteins during vitellogenesis. *Indian J. Exp. Biol.* **6:** 144–147.

ADIYODI, K. G., AND R. G. ADIYODI. 1970. Endocrine control of reproduction in decapod crustacea. *Biol. Rev.* **45:** 121–165.

ANILKUMAR, G. 1980. Reproductive physiology of female crustaceans. Ph.D. Thesis, University of Calicut, Kerala, India.

ANILKUMAR, G., AND K. G. ADIYODI. 1980. Ovarian growth induced by eyestalk ablation during the prebreeding season, is not normal in the crab, *Paratelphusa hydrodromous* (Herbst). *Int. J. Invert. Reprod.* **2:** 95–105.

BOMIRSKI, A., AND E. KLEK. 1974. Action of eyestalks on the ovary in *Rithropanopeus harrisii* and *Crangon crangon* (Crustacea, Decapoda). *Mar. Biol.* **24:** 329–337.

CHANG, E. S., AND J. D. O'CONNOR. 1983. Metabolism and transport of carbohydrates and lipids. Pp. 263–287 in *The Biology of Crustacea*, Vol. 5, L. H. Mantel, ed. Academic Press, New York.

CHEUNG, T. S. 1969. The environmental and hormonal control of growth and reproduction in the adult female stone crab, *Menippe mercenaria* (Say). *Biol. Bull.* **136:** 327–346.

CLAYBROOK, D. L. 1983. Nitrogen metabolism. Pp. 163–213 in *The Biology of Crustacea*, Vol. 5, L. H. Mantel, ed. Academic Press, New York.

DUBOIS, M., K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28:** 350–356.

HINSCH, G. W. 1972. Some factors controlling reproduction in the spider crab, *Libinia emarginata*. *Biol. Bull.* **143:** 358–366.

HOFFMANN, J. A., M. LAGUEUX, C. HETRU, M. CHARLET, AND F. GOLTZENE. 1980. Ecdysone in reproductively competent female adults and in embryos of insects. Pp. 431–465 in *Progress in Ecdysone Research: Developments in Endocrinology*, Vol. 7, J. A. Hoffmann, ed. Elsevier/North-Holland, Biomedical Press.

JOHNSTON, M. A., AND P. S. DAVIES. 1972. Carbohydrates of the hepatopancreas and blood tissues of *Carcinus*. *Comp. Biochem. Physiol.* **41B:** 433–445.

KURUP, K. N. P., AND R. G. ADIYODI. 1984. Multiple limb autotomy can trigger either ovarian growth or somatic growth in the freshwater crab, *Paratelphusa hydrodromous* (Herbst). *Gen. Comp. Endocrinol.* **56:** 433–443.

LEE, Y. P., AND T. TAKAHASHI. 1966. An improved colorimetric determination of amino acids with the use of ninhydrin. *Anal. Biochem.* **14:** 71–77.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

MCCARTHY, J. F., AND D. M. SKINNER. 1977. Proecdysial changes in serum ecdysone titers, gastrolith formation, and limb regeneration following molt induction by limb autotomy and/or eyestalk removal in the land crab, *Gecarcinus lateralis*. *Gen. Comp. Endocrinol.* **33:** 278–292.

SKINNER, D. M., AND D. E. GRAHAM. 1972. Loss of limbs as a stimulus to ecdysis in Brachyura (true crabs). *Biol. Bull.* **143:** 222–223.

VOGEL, A. I. 1959. *A Textbook of Practical Organic Chemistry Including Quantitative Organic Analysis*. Spottiswoode, Ballantyne and Co. Ltd., London.